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Rsp5, a Ubiquitin-Protein Ligase, Is Involved in Degradation of the Single-Stranded-DNA Binding Protein Rfa1 in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, *RAD1* and *RAD52* are required for alternate pathways of mitotic recombination. Double-mutant strains exhibit a synergistic interaction that decreases direct repeat recombination rates dramatically. A mutation in *RFA1*, the largest subunit of a single-stranded DNA-binding protein complex (RP-A), suppresses the recombination deficiency of *rad1 rad52* strains (J. Smith and R. Rothstein, Mol. Cell. Biol. 15:1632–1641, 1995). Previously, we hypothesized that this mutation, *rfa1-D228Y*, causes an increase in recombinogenic lesions as well as the activation of a *RAD52*-independent recombination pathway. To identify gene(s) acting in this pathway, temperature-sensitive (ts) mutations were screened for those that decrease recombination levels in a *rad1 rad52 rfa1-D228Y* strain. Three mutants were isolated. Each segregates as a single recessive gene. Two are allelic to *RSP5*, which encodes an essential ubiquitin-protein ligase. One allele, *rsp5-25*, contains two mutations within its open reading frame. The first mutation does not alter the amino acid sequence of Rsp5, but it decreases the amount of full-length protein in vivo. The second mutation results in the substitution of a tryptophan with a leucine residue in the ubiquitination domain. In *rsp5-25* mutants, the UV sensitivity of *rfa1-D228Y* is suppressed to the same level as in strains overexpressing Rfa1-D228Y. Measurement of the relative rate of protein turnover demonstrated that the half-life of Rfa1-D228Y in *rsp5-25* mutants was extended to 65 min compared to a 35-min half-life in wild-type strains. We propose that Rsp5 is involved in the degradation of Rfa1 linking ubiquitination with the replication-recombination machinery.

RAD1 and *RAD52* are involved in different pathways of mitotic recombination in yeast (19, 39, 56). Although loss of function of neither *RAD1* nor *RAD52* alone has a significant effect on most mitotic direct repeat recombination assays, *rad1 rad52* double mutants exhibit a synergistic interaction that decreases the recombination rates dramatically (19, 39, 56). This result has led to the hypothesis that *RAD1* and *RAD52* act in two alternate recombination pathways.

Strains that are mutated for *RAD1* are highly sensitive to UV light and are completely defective in the incision step of excision repair of damaged DNA (12, 35). In vivo Rad1 and Rad10 form a stable complex that exhibits a single-stranded DNA endonuclease activity (57). The function of this complex is to remove the 3′ nonhomologous regions of single-strand DNA that interfere with annealing and/or strand invasion during mitotic recombination. Consequently, *rad1* cells cannot efficiently complete recombination when the ends of the double-strand break contain approximately 60 bp of nonhomology (9). Efficient recombination is restored when the ends of the break are homologous to the donor sequences or less than 40 bp (30).

RAD52 was identified as a mutation that confers extreme sensitivity to gamma irradiation and to methyl methanesulfonate (33). Genetic analysis has revealed that wild-type *RAD52* function is required to repair double-strand breaks (23, 29, 34). More importantly, Rad52 is a conserved DNA binding protein and promotes DNA strand annealing (26, 52). It interacts physically with Rad51, a RecA homolog, that has been shown to catalyze strand exchange (25, 42, 51, 53). Recently, it was demonstrated that Rad52 stimulates Rad51-dependent strand exchange reactions and that the binding of Rad52 to Rad51 is necessary for this stimulatory effect (3, 27, 43).

A classical genetic approach was taken to study *rad1 rad52* double mutants (7). A suppressor mutation, *rfa1-D228Y*, was identified that restores wild-type levels of direct repeat recombination in *rad1 rad52* strains (45). The wild-type *RFA1* gene encodes the largest component of an essential three-subunit complex, replication factor A (4). Its human homolog is required for the initiation and elongation steps of in vitro simian virus 40 replication, as well as for excision repair (1, 5, 8). The *rfa1-D228Y* allele on its own causes a 15-fold increase in direct repeat recombination levels. This hyper-recombination phenotype is *RAD52* independent and only partially dependent on the *RAD1* gene product. In addition, the mutant strains display increased UV sensitivity and slow growth. Overexpression of the mutant protein in *rfa1-D228Y* strains results in partial suppression of the recombination defect and complete suppression of the UV sensitivity phenotype. Interestingly, the amount of RP-A complex present in extracts from *rfa1-D228Y* cells is reduced twofold compared to the amount present in wild-type cells. Taken together, these results suggest that the mutant RP-A complex is unstable and that this instability can be compensated for by overexpression of mutant Rfa1 (45).

To define further the *rfa1-D228Y*-dependent recombination pathway, *rad1 rad52 rfa1-D228Y* triple-mutant strains were screened for new mutations that decrease recombination. Here, we report that three temperature-sensitive (ts) mutations were isolated. Cloning and sequencing analyses showed that two (*rsp5-25* and *rsp5-26*) are allelic to *RSP5*, a ubiquitin-

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TABLE 1. Strains used in this study

Strain[a]	Genotype	Source
W1588-4A	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5*	Rothstein lab
W1100-3B	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 SUP4-***o***::CAN1-URA3::sup4*$^+$	Rothstein lab
W1088-1A	*MATα ade2-1 can1-100 his5Δ leu2-3,112 trp1-1 ura3-1*	Rothstein lab
W1088-10D	*MATα ade2-1 can1-100 his5Δ leu2-3,112 trp1-1 ura3-1*	Rothstein lab
W1698-11C	*MATα ade2-1 can1-100,x his3-11,15 leu2-3, 112 trp1-1 ura3-1 RAD5 rad1::HIS5 rad52::HIS5 rfa1-D228Y SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
J709	*MATα ade2-1 can1-100,x his3-11,15 leu2-3, 112 trp1-1 ura3-1 RAD5 rad1::HIS5 rad52::HIS5 rfa1-D228Y rsp5-25 SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
J714	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rad1::HIS5 rad52::HIS5 rfa1-D228Y rsp5-26 SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
W1713-1B	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rsp5-25 SUP4-***o***::CAN1-HIS3::sup4*$^+$	This study
W1713-22D	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rad1::HIS5 rad52::HIS5 rfa1-D228Y rsp5-25 SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
U1025	*MAT***a**/*α ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rsp5Δ SUP4-***o***::CAN1-HIS3::sup4*$^+$	This study
W1713-1A	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rfa1-D228Y SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
W2013-4B	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rsp5-Y647och SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
W2012-2D	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rsp5-W650L SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
W1713-22A	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rfa1-D228Y rsp5-25 SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
W2013-11C	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rfa1-D228Y rsp5-Y647ochSUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
W2012-15C	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rfa1-D228Y rsp5-W650L SUP4-***o***::CAN1-HIS3::sup4*$^+$ *leu2ΔEcoRI::URA3::leu2BstEII*	This study
W2015-1A	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rfa1Δ rsp5-25 SUP4-***o***::CAN1-HIS3::sup4*$^+$ *pWJ709-GAL1-rfa1-D228Y*	This study
W2015-4A	*MATα ade2-1 can1-100,x his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 rfa1Δ SUP4-***o***::CAN1-HIS3::sup4*$^+$ *pWJ709-GAL1-rfa1-D228Y*	This study
W2016-1B	*MATα ade2-1 can1-100,x his3-11, 15 leu2-3, 112 trp1-1 ura3-1 RAD5 rfa1Δ SUP4-***o***::CAN1-HIS3::sup4*$^+$ *pWJ708-GAL1-RFA1*	This study
CMY763	*MATα cim3-1 ura3-52 leu2Δ1*	Carl Mann
CMY765	*MATα cim5-1 ura3-52 leu2Δ1 his3Δ-200*	Carl Mann
CMY826	*MAT***a** *ura3-52 leu2Δ1 his3Δ-200 trp1Δ63 lys2-801 ade2-101 bar1::HIS3*	Carl Mann

[a] All strains used are derivatives of W303-1B (55). The genotypes are identical to that of W303-1B except where noted. In most instances several strains with the same genotype (except the mating type) were used. However, only one strain is noted. In addition, *can1-100,x* is an allele of *can1-100* in which an unidentified secondary mutation prevents suppression by *SUP4-***o.**

protein ligase. *RSP5*, repressor of *spt3* phenotype, was first isolated as a suppressor of Spt3, a subunit of the SAGA complex (36). Subsequently, *rsp5* mutations have been observed to affect many diverse cellular processes. These include stability of both the largest subunit of RNA polymerase II, Rpb1, and the uracil permease, Fur4 (11, 18). Also, it was shown that both the localization of Mod5 (a tRNA modifier) and the mitogen-activated protein kinase cascade are affected by *rsp5* mutations (59, 62). One of the alleles described here, *rsp5-25*, contains two changes in its open reading frame (ORF). The first changes a tyrosine codon to an ochre codon, resulting in the truncation of the full-length protein. In the genetic background used for the screen, the ochre mutation is partially suppressed by the tyrosine-inserting tRNA suppressor, *SUP4-***o**. This mutation does not create a change in the amino acid sequence of Rsp5; however, it causes a decrease in the level of full-length protein in vivo. The second mutation results in the substitution of a tryptophan residue with a leucine residue in the ubiquitination domain. Biochemical and genetic studies show that Rfa1-D228Y is stabilized in *rsp5-25* mutant strains. Thus, the increased amount of Rfa1-D228Y protein causes a decrease in the recombination levels of *rad1 rad52 rfa1-D228Y* strains. We hypothesize that Rsp5 is involved in the degradation of Rfa1, thereby linking ubiquitin-dependent protein degradation to the replication-recombination machinery.

MATERIALS AND METHODS

Media, strains, and genetic methods. All media were prepared as described previously (24, 45). Standard genetic techniques were employed (41). All yeast strains are derivatives of a *RAD5* W303-1B (46, 55) unless otherwise noted and are listed in Table 1. W303 derivatives containing the *SUP4* duplication, *leu2* direct repeats, and *rad1::HIS5*, *rad52::HIS5*, *rfa1-D228Y*, and *can1-100,x* mutations were described earlier (24, 45, 46). A modified version of the *SUP4* construct was used in this study (U928). The *URA3* gene flanked by the *SUP4* repeats was disrupted by the *HIS3* gene. Lastly, *cim3 cim5* strains (the 26S proteasome mutants) and their congenic wild-type strain were gifts from C. Mann.

The *rfa1-D228Y* mutation creates a novel *Acc*I restriction site, which permits the segregation of this allele to be monitored by PCR analysis by using the *RFA1*-2A (5′-CAGAGCATCCAAATGAAACC-3′) and the *RFA1*-3B (5′-TTT GGATAATACCGAGGACG-3′) primers as described earlier (45). The presence of *rad1::HIS5* and *rad52::HIS5* alleles were identified by complementation test for their respective UV and X-ray sensitivity phenotypes.

In this study, a novel allele of *RSP5* was isolated and named *rsp5-25*. The *rsp5-25* allele was scored in two ways: the presence of the mutation was confirmed by crosses to a *rad1 rad52 rfa1-D228Y* strain and determining whether lower recombination frequency and a ts phenotype is observed in half of the *rad1 rad52 rfa1-D228Y* segregants. Later, we found that the *rsp5-25* allele contains an additional *Mun*I restriction enzyme site not present in the wild-type *RSP5* sequence. Therefore, it is easily detected by colony PCR by using *RSP5*-G (5′-G AAGGTGTTGACGCAGAGGTG-3′) and *RSP5*-H (5′-CCGCAATACCACC GATCAATAG-3′) primers and a diagnostic *Mun*I restriction enzyme digestion. To perform linkage analysis of *RSP5* and *mrr1*, a 5.9-kb *Sal*I fragment of the complementing library insert was cloned into an integrating vector, YIp5. The plasmid was linearized by *Bgl*II digestion, which cuts uniquely in the *Sal*I fragment, and transformed into a wild-type strain (W1588-4C). As a result, the 5.9-kb region was duplicated and marked with the *URA3* gene in the genome (U957).

Strains containing only the ochre mutation (*rsp5-Y647och*) or only the missense mutation (*rsp5-W650L*) of *rsp5-25* were constructed by using a PCR-based allele replacement method as described previously (20). The primers used for the construction of *rsp5-Y647och* strain were Stop1 (5′-GGTAATAAGAAAGAATATGTCGAATTATAAACCCAATGGAGAATTGTTGATAGAGTTCAAG*TTTTCCCAGTCACG*-3′) and Stop2 (5′-TTGAACTCTATCAACAATTCTCCATTGGGTTTATAATTCGACATATTCTTTCTTATTACC*GGATCCTCTAGAGTCG*-3′). Similarly, the primers for creating the *rsp5-W650L* allele were WtoL1 (5′-AGAAAGAATATGTCGAATTATATACCCAATTGAGAATTGTTGATAGAGTTCAAGAACAATG*TTTTCCCAGTCACG*-3′) and WtoL2 (5′-AATGTTCTTGAACTCTATCAACAATTCTCAATTGGGTATATAATTCGACATATTCTTTCT*GGATCCTCTAGAGTCG*-3′). Each pair of 75-mer oligonucleotides were constructed so that the last 15 nucleotides of the oligonucleotides (italics) were used to amplify *URA3* from pUC18-*URA3*. The remaining nucleotides of both primers are complementary to each other and contain the desired mutation (underlined letter) in the middle of 60 nucleotides of the *RSP5* gene. After PCR amplification, a linear fragment was transformed into a diploid strain that contains the *SUP4* construct. The Ura$^+$ transformants were screened for the desired integration events by PCR with an internal *URA3* primer (5′-CAACACTACATATGCG-3′) and the *RSP5*-H primer (5′-CCGCAATACCACCGATCAATAG-3′), which only give a product if the construct integrates at the *RSP5* locus. Transformants integrated into the *RSP5* locus for *rsp5-Y647och* and for *rsp5-W650L* mutations were identified (J717 and J718, respectively). After transformation with a plasmid that contains the wild-type *RSP5* gene, these diploid strains were sporulated and dissected. The Ura$^+$ segregants were plated on 5-fluoro-orotic acid medium to select for direct repeat recombinants that "pop-out" the *URA3* gene. The *rsp5-Y647och* strains were verified by their inability to lose the *SUP4*-**o** allele, since the ochre mutation causes a truncation in an essential protein and requires *SUP4*-**o** for viability. The presence of the *rsp5-W650L* allele was identified by PCR analysis for a *Mun*I restriction polymorphism as described above.

The *RSP5* gene disruption was constructed in a diploid strain (W1588) by a PCR-based gene disruption method utilizing *TRP1* as the selective marker (2). The following primers were used for disruption: *RSP5*-T1 (5′-ATGCCTTCATCCATATCCGTCAAGTTAGTGGCTGCAGAGTCATTATATAAGAGGGACGTA*TTTCCCAGTCACGACG*-3′) and *RSP5*-T2 (5′-TCATTCTTGACCAAACCCTATGGTTTCTTCCACGGCCAATGTTAGCTTCTGTTTCATGCT*GCAAGTGCACAAACAAT*-3′). The italicized sequences are those that are required to amplify the *TRP1* gene. Three Trp$^+$ transformants were obtained after the transformation. Disruption of the *RSP5* locus was confirmed by genomic blot analysis.

Mutagenesis. A *rad1 rad52 rfa1-D228Y* strain with *SUP4* and *leu2* direct repeat constructs (W1698-11C) was mutagenized with 0.3% ethyl methanesulfonate to 10% survival as described previously (45). Next, 200,000 mutagenized colonies were grown at 23°C and were first screened for temperature sensitivity at 37°C. A total of 2,347 ts mutants were further tested for decreased recombination by replica plating onto canavanine-containing medium at 30°C. Recombination frequencies of mutants were measured by both *SUP4* and *leu2* direct repeats constructs as described previously (45, 46).

Plasmid constructions. The plasmid pWJ611 containing *RAD1*, *RAD52*, and *RFA1* used for complementation was created in three steps. First, a 2.5-kb *Pst*I-*Hin*dIII fragment of *RFA1* was end filled by Klenow polymerase, and *Bam*HI linkers were added. This fragment was cloned into the *Bam*HI site of pRS414 (44) to create pWJ609. A 3.3-kb *Sal*I fragment that contains full-length *RAD52* was cloned into the *Sal*I site of pWJ609 to generate pWJ610. Lastly, a 5.9-kb Klenow end-filled *Sal*I fragment of *RAD1* was cloned into the *Sma*I site of pWJ610 to create pWJ611.

The *rsp5-25* complementing clone (pWJ670) was isolated from a library of *Sau*3A-digested yeast genomic DNA fragments cloned into YCp50 (38) and was shown by DNA sequence and restriction digest analyses to contain five ORFs (*GLO3*, *YCK3*, *YER124*, *RSP5*, and *YER126*). For linkage analysis, a 5.9-kb *Sal*I fragment containing part of *GLO3*, as well as full-length *YCK3* and *YER124*, was inserted into the *Sal*I site of an integrating vector, YIp5 (48). As shown in Fig. 2B, three *Cla*I-*Cla*I fragments of 1.3, 2.5, and 4.9 kb were deleted to remove *YCK3*, *YER124*, *RSP5*, and *YER126* (plasmid 1). To delete *YER124*, *RSP5*, and *YER126*, pWJ670 was also digested with *Eco*RV and religated (plasmid 2). The 5.9-kb *Sal*I fragment from pWJ670 was ligated into the corresponding site in YCp50 (plasmid 3). In pWJ671, a 6.0-kb *Hin*dIII-*Hin*dIII fragment was deleted from pWJ670 to remove *GLO3*, *YCK3*, and *YER124*. In pWJ914, a 2.0-kb *Bst*EII-*Bst*EII fragment is deleted from pWJ671 so that most of the *RSP5* ORF is also removed.

pWJ708 and pWJ709 were constructed by cloning the wild-type *RFA1* and *rfa1-D228Y* alleles, respectively, into pYX243 plasmid (kindly provided by Morten Dunø). The *RFA1* alleles were amplified by PCR by using *RFA1*BH5 (5′-CGAGGATCCTATGAGCAGTGTTCAACTTTC-3′) and *RFA1*BH3 (5′-CGAGGATCCGCTAACAAAGCC-3′). *Bam*HI sites were introduced by PCR primers to permit the in-frame fusion of hemagglutinin (HA) tags at the C termini of *RFA1* and *rfa1-D228Y* ORFs to aid in detection of Rfa1 protein by the HA antibody during protein blot analysis.

Mapping and sequencing of *rsp5-25*. The mutations in the *rsp5-25* allele were localized by a gap repair experiment (28). pWJ671 was digested with various combinations of the following enzymes: *Pml*I, *Sna*BI, *Bst*EII, and *Xba*I to create several overlapping gaps in the *RSP5* ORF (the thick lines in Fig. 3A). These linear plasmid fragments were transformed into W1713-1B, an *rsp5-25* strain, and repaired circular plasmids were selected as Ura$^+$ transformants. After rescue in *Escherichia coli*, several gap-repaired plasmids were retransformed into W1713-22D, a *rad1 rad52 rfa1-D228Y rsp5-25* strain, to test for complementation. A 395-bp region between the *Sna*BI and *Bst*EII sites in *RSP5* was identified that contains the *rsp5-25* mutation. Both mutant and wild-type plasmids were sequenced by using *RSP5*-G and *RSP5*-H primers.

Analysis of UV sensitivity. For any given genotype, UV sensitivity was determined by analyzing three segregants three times as described previously (45).

Analysis of degradation kinetics of Rfa1-D228Y and immunoprecipitations. Both *RSP5* (W2015-1A) and *rsp5-25* (W2015-4A) strains with the plasmid that contains the *rfa1-D228Y* gene under the control of *GAL1* promoter were grown to 1×10^7 to 2×10^7 cells/ml in galactose liquid medium lacking leucine. Next, the cells were washed and resuspended in rich liquid medium (YPD liquid). For the S-phase experiments, 100 mM hydroxyurea (HU) was added to an early-log-phase culture for 6 h at 30°C. Next, the cells were washed and resuspended in YPD liquid medium with 100 mM HU. For both HU-arrested and nonarrested cultures, samples were taken every 15 and 30 min, respectively, for 3 h at 30°C.

Total protein extract was prepared from each sample by using glass bead disruption as described earlier (14). Then, 15 μg of total protein (determined by using the Bio-Rad Protein Concentration Assay) were separated by electrophoresis with sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (Bio-Rad). The extracts were transferred to polyvinylidene difluoride membranes, and protein blot analysis was performed as described previously (45). The resulting bands on Kodak X-Omat films were quantitated by using a computing densitometer (model 300A; Molecular Dynamics). The exponential decay of concentration with time exhibited first-order reaction kinetics and was used to calculate the relative half-lives. The relative half-life of each genotype was determined by averaging the results from 9 to 12 trials. One representative of each is shown in Fig. 6A. It is important to note that the observed decay is a function of protein half-life, as well as mRNA half-life.

The levels of Rsp5 protein present in different *RSP5* backgrounds were determined by protein blot analysis as described above. The blots were incubated with monoclonal antibody raised against Rsp5, a kind gift from J. Huibregste.

cim3 and *cim5* mutant strains that overexpress *rfa1-D228Y* under the *GAL1* promoter were used for the pulse-chase experiments as described previously (13). Immunoprecipitations were performed as described previously (4). After separation of the immunoprecipitates on SDS–10% polyacrylamide gels, the gels were processed with the Entensify Kit (DuPont) according to the manufacturer's instructions.

RESULTS

Isolation of mutations that reduce the recombination levels of *rad1 rad52 rfa1-D228Y* strains. To identify mutations that decrease the recombination levels of *rad1 rad52 rfa1-D228Y* strains, two direct repeat recombination constructs were employed. In each assay, decreased recombination is indicated by fewer papillae in the selective medium. Each assay was chosen to utilize different metabolic pathways to avoid specifically mutations that affect a metabolic pathway. The first construct is a nontandem *leu2* direct repeat consisting of two *leu2* mutant alleles, *leu2*-Δ*Eco*RI and *leu2*-Δ*Bst*EII, that are separated from each other by *E. coli* plasmid sequences and a yeast selectable marker, *URA3* (Fig. 1A). Direct repeat recombination events that generate a single, wild-type *LEU2* allele and delete the intervening sequences are selected first on medium lacking leucine and screened for uracil auxotrophy.

The second construct utilizes direct repeats of a yeast tyrosine tRNA gene where one copy is wild type (*sup4*) and the other copy is an ochre suppressor (*SUP4*-**o**), as depicted in Fig. 1B (24, 37). These two alleles differ at a single position in the anticodon. The repeats are separated from each other by plasmid sequences and two yeast selectable markers, *CAN1* and *HIS3*. The *CAN1* gene encodes the arginine permease, which allows the uptake of canavanine, an arginine analog and a cell poison (16). Cells that undergo a deletion event between the *SUP4* alleles become Can^r and His$^-$ and are selected on canavanine-containing medium.

For both the *leu2* and the *SUP4* constructs, several possible recombination mechanisms can be envisioned to lead to the retention of a single repeat. Such mechanisms include intrachromosomal recombination, unequal sister chromatid exchange, unequal sister chromatid gene conversion, replication

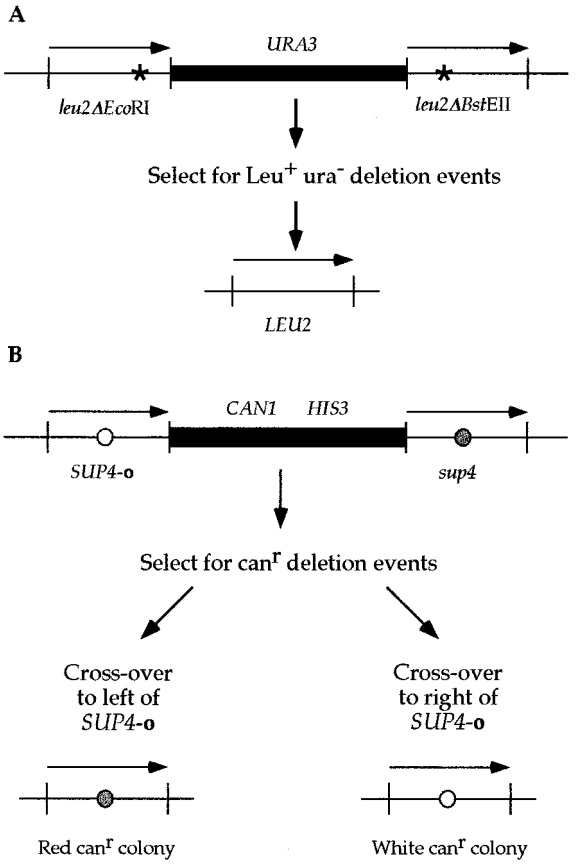

FIG. 1. The direct repeat recombination constructs for *leu2* and *SUP4* are depicted. Both assays utilize a direct repeat of two alleles that are separated by plasmid and selectable marker sequences. Recombination events between the repeats that result in the retention of one allele and deletion of the intervening sequences can be selected. The *leu2* and *SUP4* direct repeats are 2.4 kb and are not drawn to scale. (A) In the *leu2* assay (46), both alleles contain a frameshift mutation created by filling in a restriction enzyme site (*Eco*RI or *Bst*EII). Leu^+ recombinants are first selected for on medium lacking leucine and further identified as Ura^- colonies after replica plating on medium lacking uracil. (B) The *SUP4* assay (24) was modified by disrupting *URA3* with *HIS3* as described in Materials and Methods. The alleles differ by a single nucleotide change in the anticodon. Can^r recombinants are selected on canavanine-containing medium. The strain contains the ochre suppressible *ade2-1* allele, which enables colony color to designate which *SUP4* allele is retained in the genome after direct repeat recombination: *sup4* colonies are red, while *SUP4*-**o** colonies remain white. Deletions are confirmed by their failure to grow on histidine-less medium.

slippage, and single-strand annealing (6, 21, 22, 24, 31, 47, 54). Neither of the two constructs allows these mechanisms to be distinguished from one another, and thus all recombination events are collectively referred to as plasmid loss. However, both constructs permit the visualization of recombination levels simply by replica plating yeast strains onto selective media. In *rad1 rad52 rfa1-D228Y* strains, typically 20 to 30 papillae are observed after the recombination event between the direct repeats (Fig. 2A). However, in *rad1 rad52* strains, the number of papillae is lowered at least 10-fold (data not shown).

We mutagenized a *rad1 rad52 rfa1-D228Y* strain to 10% survival with ethyl methanesulfonate and screened for ts mutations among 200,000 colonies, generating a collection of 2,345 ts strains. Next, these ts strains were screened for their recombination phenotype at 30°C, a semipermissive temperature. A decreased number of papillae on selective medium with both recombination constructs reflects a decrease in recombination (Fig. 2A). Three mutants consistently displayed this phenotype and were provisionally named *mrr* (mutated for *rfa1*-stimulated recombination). None of the three exhibited a specific cell cycle arrest phenotype.

Each mutant was crossed to a wild-type strain, and random spore analyses were performed. For each mutation, half of the *rad1 rad52 rfa1-D228Y* segregants were both ts and recombination-deficient, indicating that a mutation in a single gene is likely responsible for both traits and that it is unlinked to *RAD1*, *RAD52*, or *RFA1* (data not shown). Crosses to *rad1 rad52 rfa1-D228Y MRR* strains revealed that all three mutations are recessive for temperature sensitivity since the diploids are temperature resistant. Similarly, after the three mutant strains were crossed to each other, the resulting diploids were no longer ts, indicating that the *mrr* mutations define three recessive complementation groups. Each mutant displays a 5- to 10-fold reduction with the *leu2* construct and at least 40-fold reduction with the *SUP4* construct in the levels of recombination compared to the parental *MRR* strain.

To investigate the phenotype of *mrr* single mutants, a plasmid that complements the *rad1*, *rad52*, and *rfa1-D228Y* mutations, pWJ611, was transformed into each strain. After transformation, *mrr2* strains remain ts, indicating that the *mrr2* mutation on its own causes temperature sensitivity. In contrast, pWJ611-containing *mrr1* and *mrr3* strains are no longer ts. Results from genetic crosses indicated that *mrr1* strains are ts only in the absence of a functional *RAD52* gene. On the other hand, *mrr3* is ts with any combination of the *rad1*, *rad52*, or *rfa1-D228Y* mutations. Additionally, *mrr3* mutants are ts in a *rad51* mutant background. We focused on characterizing *mrr1* and *mrr3* since these two mutations display conditional lethal interactions in conjunction with other recombination mutations.

***mrr1* and *mrr3* are alleles of *RSP5*, an essential ubiquitin-protein ligase.** A centromere-based yeast library (38) was transformed into a *rad1 rad52 rfa1-D228Y mrr1* strain to identify a wild-type clone by complementation. Among 5,000 transformants screened for temperature resistance and subsequently for complementation of decreased recombination, one complementing clone was identified. To determine whether this clone encodes *MRR1*, we first demonstrated that sequences from the library clone were genetically linked to *mrr1*. Part of the insert was cloned into YIp5 as described in Materials and Methods and integrated into a wild-type strain by homologous recombination to mark the genomic integration site with *URA3*. This Ura^+ strain was crossed to a *rad1 rad52 rfa1-D228Y mrr1* strain. The resulting diploid was sporulated and dissected. Eight *rad1 rad52 rfa1-D228Y* Ura^- segregants were tested, and each displayed a ts and a low recombination phenotype, indicating the presence of *mrr1*. In contrast, eight Ura^+ *rad1 rad52 rfa1-D228Y* segregants were temperature resistant and had high levels of recombination. This demonstrates that the complementing clone is genetically linked to the wild-type *MRR1* gene.

The complementing clone contains five ORFs; therefore, a series of subclones were constructed to determine which ORF corresponds to *MRR1*. Only constructs containing the full-length *RSP5* gene complement both the ts and recombination phenotype of *rad1 rad52 rfa1-D228Y mrr1* strains (pWJ670 and pWJ671 in Fig. 2B). Next, we showed that disruption of the plasmid-based *RSP5* gene eliminated complementation, confirming that *mrr1* is an allele of *RSP5* (pWJ914 in Fig. 2B). Hereafter, the *mrr1* mutation is referred to as *rsp5-25*.

Similarly, *mrr3* was cloned by complementing its recessive ts phenotype. One of 10,000 transformants displayed temperature resistance and exhibited the recombination levels of *rad1 rad52 rfa1-D228Y* strains. DNA sequence analysis revealed

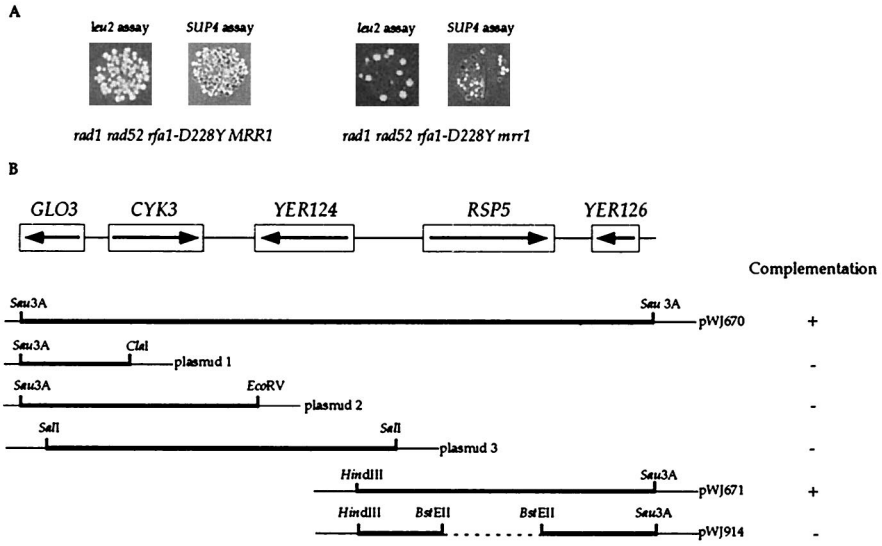

FIG. 2. Identification of *mrr* mutants and cloning of the *mrr1* mutation. (A) Papillation phenotype of the *mrr1* mutation in *rad1 rad52 rfa1-D228Y* after replica plating to the selective media for *SUP4* and *leu2* direct repeat recombination assays. (B) *mrr1* is allelic to *RSP5*. The five ORFs present in pWJ670 are illustrated, as well as the five subclones constructed from this plasmid by using the indicated restriction enzymes. Each subclone was used to determine which of the five ORFs is *MRR1*. The *Sau*3A sites represent the junction of the genomic yeast sequences and the plasmid sequences. The thin lines depict the plasmid sequences. The plasmid, pWJ914, was derived from pWJ671 and carries a deletion of an internal *Bst*EII fragment indicated as the dashed line. Each plasmid was tested for complementation of both the ts and the reduced recombination phenotypes in *rad1 rad52 rfa1-D228Y mrr1* strains, and the results are shown adjacent to each plasmid.

that the complementing plasmid also contained the *RSP5* ORF and surrounding region. By using the same strategy described for the *mrr1* above, *mrr3* was shown to be an allele of *RSP5* and was renamed *rsp5-26*. Thus, *mrr1* and *mrr3* are alleles of the same gene and display intragenic complementation. This is consistent with Rsp5 functioning as a multimer (61). Since both *mrr* mutations were allelic to each other and display a very similar phenotype, we only characterized the *rsp5-25* allele in more detail.

The defect in *rsp5-25* is due to two mutations localized in its ubiquitination domain. Plasmid gap repair was used to localize the genetic alteration in the *rsp5-25* mutant allele (28). By this analysis, a 395-bp region between the *Sna*BI and *Bst*EII enzyme sites was shown to contain the mutation(s) (Fig. 3A). DNA sequence analysis of this region revealed two changes. The first, a T-to-A transversion at position 1941 in the *RSP5* ORF, results in an ochre codon in place of a tyrosine codon (Fig. 3B) and truncates the protein from 803 to 647 amino acids. The second mutation is a T-to-G transversion at position 1950, resulting in a leucine-to-tryptophan substitution at residue 650 (Fig. 3B).

Genetic crosses reveal that, in the absence of *SUP4*-**o**, the *rsp5-25* mutation is lethal (see Materials and Methods). Such a mutation could be isolated since the parental strain contains the *SUP4*-**o** suppressor, which recognizes the ochre codon to produce full-length Rsp5. However, since suppression is only partial, an approximately 10-fold decrease in the level of full-length Rsp5 protein is observed by protein blot analysis (Fig. 4). Interestingly, ochre suppression does not create an amino acid change, since both wild-type and *SUP4*-**o**-suppressed proteins contain a tyrosine residue in this position (32).

The second mutation, the tryptophan-to-leucine substitution at position 650, is in the ubiquitination domain of Rsp5 (reference 17 and Fig. 3B). To establish whether the effect of *rsp5-25* is due to the lowered amount of Rsp5 and/or the missense mutation, we separated the two mutations from each other by a PCR-based allele transfer method (20). The ochre mutation was named *rsp5-Y647och*, and the second mutation was named *rsp5-W650L*. Since both *rsp5-Y647och* and *rsp5-25* mutations are lethal in the *sup4* background, all tests of their effects were performed in a *SUP4*-**o** background. These results will be discussed below.

Both mutations in *rsp5-25* are required to suppresses *rfa1-D228Y*. Since *rsp5-25* was isolated as a suppressor of *rfa1-D228Y*-stimulated direct repeat recombination, we next asked whether it suppresses the UV sensitivity exhibited by *rfa1-D228Y* strains. As a control, we showed that the *rsp5-25* mutant strain itself is not sensitive to UV damage (data not shown). Figure 5 shows that the UV sensitivity of *rfa1-D228Y* is partially suppressed in an *rsp5-25 rfa1-D228Y* double mutant.

Next, we determined the extent to which each separate alteration in *rsp5-25* contributes to its phenotype. All tests were performed in the presence of *SUP4*-**o**. Neither *rsp5-Y647och* nor *rsp5-W650L* on its own reduces direct repeat recombination levels in a *rad1 rad52 rfa1-D228Y* background (data not shown). Similarly, neither allele displays temperature sensitivity in a *rad52* mutant background nor can either one suppress the UV sensitivity of *rfa1-D228Y* (data not shown). Thus, both the decreased level of full-length protein caused by *rsp5-Y647och* and the *rsp5-W650L* missense mutation in the ubiquitination domain are necessary to observe the *rsp5-25* phenotype, indicating a synergistic interaction between the two mutations.

The stability of Rfa1-D228Y is increased in *rsp5-25* strains. The relative stability of Rfa1-D228Y was measured and compared in *rsp5-25* and wild-type strains. A plasmid containing a functional HA-tagged *rfa1-D228Y* allele under the control of the *GAL1* promoter was introduced into both *rsp5-25 rfa1*Δ and *RSP5 rfa1*Δ strains. These strains were grown in the presence of galactose until early log phase and then shifted to glucose-containing medium to repress the *GAL1* promoter and to turn off the production of Rfa1-D228Y. Samples were taken at 30-min intervals for 3 h, and the Rfa1-D228Y protein levels were evaluated by protein blot analysis. As shown in Fig. 6A, under these conditions the half-life of Rfa1-D228Y in the *rsp5-25* strain is 65 min compared to 35 min in the *RSP5* strain.

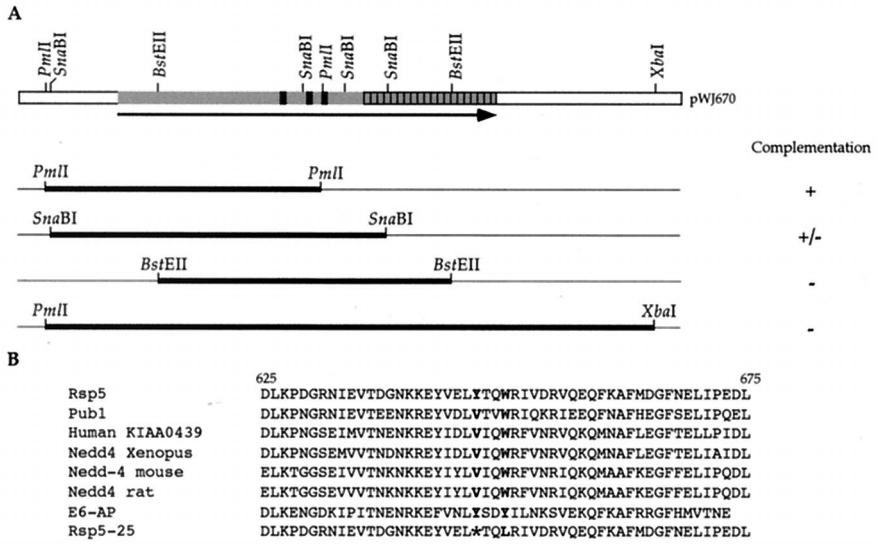

FIG. 3. Localization of the mutations in *rsp5-25*. (A) The *rsp5-25* mutation was mapped by using a plasmid that contains the wild-type *RSP5* allele, digested with indicated restriction enzymes for use in plasmid gap repair experiments. The gapped regions are indicated as thick lines. After transformation into an *rsp5-25* strain, plasmid gap repair results in the copying of the corresponding genomic region onto the plasmid. The results of complementation experiments in *rad1 rad52 rfa1-D228Y rsp5-25* are depicted. The black boxes on the *RSP5* ORF depict the WW domains, and the hatched box represents the ubiquitination domain of the protein. (B) Localization of the two *rsp5-25* mutations in the ubiquitination domain. The mutated tryptophan residue is indicated as a boldface letter, and the ochre codon is indicated by an asterisk. The protein sequences of several Rsp5 homologs are also shown to indicate the conservation near the mutated residues.

Furthermore, Fig. 6B shows that the same steady-state levels of Rfa1-D228Y protein is seen in *rfa1-D228Y rsp5-25* strains and *rfa1-D228Y* strains where *rfa1-D228Y* is overexpressed.

Next, the protein levels of Rfa1-D228Y were measured during the cell cycle, where *RFA1* mRNA levels have been shown to increase fourfold at the beginning of S phase (reference 5 and Fig. 6C). Surprisingly, Rfa1-D228Y protein levels remain constant throughout the cell cycle (Fig. 6D). Similar results have been observed in *RFA1* wild-type strains (reference 5 and data not shown). This suggests that either regulation at the level of translation and/or a faster rate of degradation of Rfa1 during S phase may account for the constant steady-state protein levels. We tested the second possibility by measuring the degradation kinetics of both wild-type Rfa1 and Rfa1-D228Y proteins in S-phase-arrested cells. Cells were arrested by incubation with HU for 6 h in the presence of inducer (galactose). Next, they were switched to glucose medium, still in the presence of HU, and the half-lives of the Rfa1 proteins were determined by protein blot analysis. In HU-arrested cells, wild-type and mutant Rfa1 proteins were shown to have approximately 65- and 35-min half-lives, respectively (Fig. 7A and B), which is not different from the findings with unarrested cells. Thus, it is more likely that the difference in mRNA and protein levels during S phase is due to regulation at the level of translation.

Rfa1 may be ubiquitinated in vivo. The observation that a mutation in Rsp5 destabilizes Rfa1 suggests that its turnover may be controlled by ubiquitination. Therefore, we examined Rfa1 for ubiquitin conjugates in vivo. Total Rfa1 protein was immunoprecipitated with Rfa1 antibodies from a strain containing *cim3* and *cim5* ts mutations that affect 26S proteasome

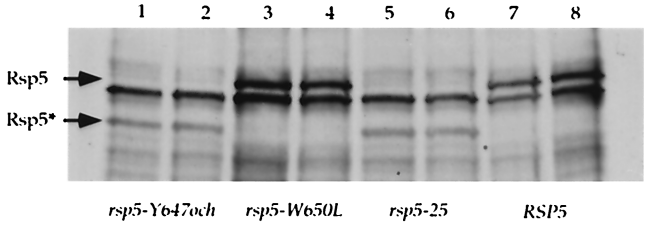

FIG. 4. Protein blot analysis of different *RSP5* alleles. Extracts from two *rsp5-25*, *rsp5-Y647och*, *rsp5-W650L*, and *RSP5* strains were prepared, and the total amount of Rsp5 protein was detected by protein blot analysis by using Rsp5 antibody. The full-length Rsp5 proteins (92 kDa) is indicated as Rsp5. In *rsp5-25* and *rsp5-Y647och* strains, an additional truncated form of Rsp5 (72 kDa) was observed and is indicated as Rsp5*. This is due to the ochre codon present at the position of residue 647. The *SUP4-o* suppressor also present in these strains recognizes the ochre codon to produce full-length Rsp5. However, since *SUP4-o* suppression is only partial, both full-length and truncated forms of Rsp5 are observed. The other bands are background obtained with the Rsp5 antibody.

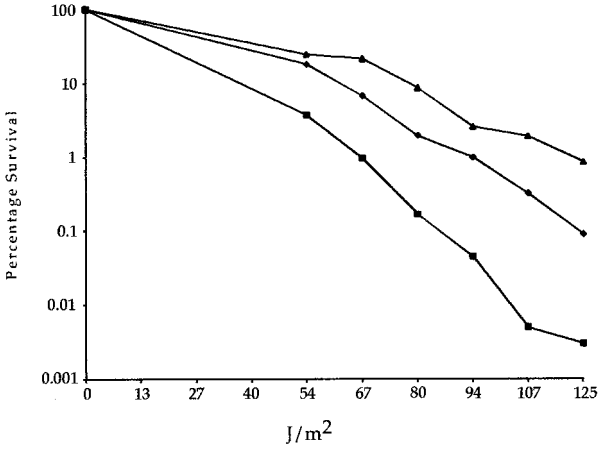

FIG. 5. UV survival curves. Wild-type (▲), *rfa1-D228Y* (■), and *rfa1-D228Y rsp5-25* (◆) strains were exposed to increasing amounts of UV irradiation, and their survival was plotted. Both *rsp5-25* strains and *rfa1-D228Y* strains overexpressing *rfa1-D228Y* exhibit identical survival to wild-type strains (reference 45 and data not shown).

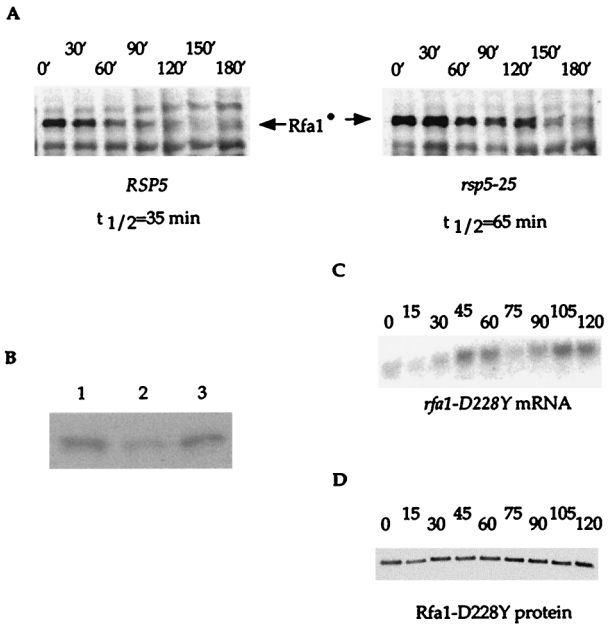

FIG. 6. Protein stability and mRNA levels of *rfa1-D228Y*. (A) A protein blot analysis of the stability of Rfa1-D228Y in the *RSP5* and *rsp5-25* mutant strains was performed. Rfa1-D228Y protein is tagged with HA and is indicated on the blots as Rfa1•. Other bands are due to background obtained with the HA antibody. The half-lives were calculated as described in Materials and Methods. (B) Protein levels of Rfa1-D228Y in *rfa1-D228Y* strains overexpressing *rfa1-D228Y* (lane 1) and *rfa1-D228Y rsp5-25* strains (lane 3) were compared by a protein blot analysis. *rfa1-D228Y* strains (lane 2) were included as a control. (C and D) Measurement of mRNA and protein levels in *rfa1-D228Y* strains during the cell cycle. To measure mRNA and protein levels of *rfa1-D228Y* during the cell cycle, cells were released from alpha-factor arrest, samples were taken every 15 min, total mRNA was extracted, and total yeast protein extracts were prepared as described in Materials and Methods. (C) RNA blots were performed by using an internal fragment of *RFA1* as a probe and *URA3* as a loading control. The mRNA levels for *URA3* were unchanged (data not shown). (D) Protein blot analysis was performed by using Rfa1 antibody. As a control, the same blot was reprobed with Rsp5 antibody. No variation in Rsp5 protein levels was detected (data not shown). After release from arrest, cells with small buds started to appear at 30 min and peaked at 45 min for the first cell cycle. The peak for cells with small buds for the second cell cycle occurs at 105 min.

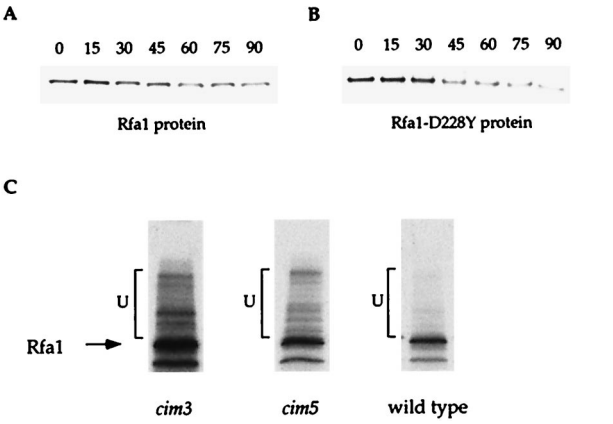

FIG. 7. Degradation of Rfa1 during S phase and its in vivo ubiquitination. (A and B) Protein blot analyses of the stability of Rfa1 and Rfa1-D228Y during S phase. *RFA1* and *rfa1-D228Y* cells were arrested with HU at S phase, and samples were taken every 15 min. Total yeast protein extracts were prepared and subjected to protein blot analysis by using the Rfa1 antibody. The half-lives of each were identical to that determined for logarithmically growing cells (see Fig. 6A). (C) In vivo ubiquitination of Rfa1. Wild-type, *cim3*, and *cim5* mutant strains that overexpress *rfa1-D228Y* under the *GAL1* promoter were pulse labeled with [^{35}S]methionine. After incubation at 37°C, the nonpermissive temperature for the *cim* mutants, total yeast protein extract was prepared, and immunoprecipitations were performed with Rfa1 antibody. By immunoprecipitation, putative ubiquitinated forms of Rfa1 were visualized in *cim3*, *cim5*, and wild-type strains respectively. The bands corresponding to the nonubiquitinated forms of the Rfa1 are indicated. The higher-molecular-weight bands, indicated by brackets, represent the putative ubiquitinated forms.

function (13). In these strains, degradation of ubiquitinated proteins in the 26S proteasome is impaired and, at the restrictive temperature, substrates accumulate with branched ubiquitin chains. To visualize potential ubiquitinated forms of Rfa1, both *cim3* and *cim5* strains expressing wild-type Rfa1 endogenously and Rfa1-D228Y from a plasmid were labeled with [^{35}S]methionine in a pulse-chase experiment. After the shift to the restrictive temperature, total Rfa1 was immunoprecipitated. Upon gel electrophoresis, a ladder of higher molecular weight forms of Rfa1 protein was detected, a finding that is consistent with heterogeneous ubiquitination (Fig. 7C).

DISCUSSION

Ubiquitin-dependent proteolysis is a major pathway for protein degradation. It functions to regulate many cellular processes by modulating the availability of proteins in the cell (reviewed in reference 15). Ubiquitin, a very conserved 76-amino-acid peptide, marks proteins for degradation when it is covalently attached to them. Degradation occurs mainly in the 26S proteasome but also occasionally in the vacuole. A key group of enzymes of this pathway are the ubiquitin-protein ligases. Their role is to recognize substrates and to promote the transfer of ubiquitin, directly or indirectly, onto the substrates, thus targeting them for degradation (15).

In our search for mutations that reduce recombination levels in *rad1 rad52 rfa1-D228Y* strains, we isolated two alleles of *RSP5*, a gene encoding an essential protein ubiquitin ligase in *S. cerevisiae* (17, 58). In the present study we extensively characterized one of these alleles, *rsp5-25*. We observed that many aspects of the *rsp5 rfa1-D228Y* phenotype could be mimicked by overexpressing *rfa1-D228Y*. First, the fivefold reduction in *leu2* direct repeat recombination observed in *rad1 rad52 rfa1-D228Y rsp5-25* strains is similar to the 4.2-fold reduction seen when the Rfa1-D228Y protein is overexpressed in *rad1 rad52 rfa1-D228Y* strains (unpublished data). Similarly, the level of suppression of UV sensitivity in *rfa1-D228Y rsp5-25* strains is the same as that seen when Rfa1-D228Y is overproduced in an *rfa1-D228Y* strain (45; Fig. 5). Third, the protein levels of Rfa1-D228Y in those same strains is increased to approximately the same levels (Fig. 6B). Furthermore, the half-life of Rfa1-D228Y is doubled in *rsp5-25* strains (Fig. 6A). Finally, the ubiquitination and degradation of Rfa1 likely occurs in the 26S proteasome in vivo (Fig. 7C). Taken together, these results implicate Rsp5 in the degradation of Rfa1, a protein involved in DNA repair, recombination, and replication.

Molecular characterization revealed that the *rsp5-25* allele contains two nucleotide changes. The first mutation causes a tyrosine-to-ochre codon change at position 647, thus truncating the protein in a *sup4* background. In contrast, in a *SUP4*-**o** tyrosine ochre suppressor background, tyrosine is inserted as in the wild-type protein. However, suppression by *SUP4*-**o** is only partial; thus, the amount of the full-length Rsp5 protein is reduced approximately 10-fold. The second mutation substitutes a leucine residue for a conserved tryptophan residue at position 650. The two mutations were separated from each other, and both mutations are required in order to observe the *rsp5-25* phenotype. Thus, the decreased level of full-length

protein as well as the missense mutation in the conserved domain are necessary to alter the degradation kinetics of Rfa1-D228Y.

Although the consequences of *rsp5* mutations on a variety of cellular processes are dramatic, their effects on the protein stability of known substrates (Fur4, Rfa1, and Rbp1) are only increased two- to fivefold (11, 18; this study). We offer several hypotheses to explain this observation. First, an even greater increase in stability may be observed in some alleles; however, such mutants may be lethal and thus could not be recovered. Second, cells may not be able to tolerate an increase in the stability of certain proteins, especially if these proteins are found in complexes. One example is the RP-A complex itself, where overexpression of only one RP-A subunit causes slow growth (5), which is likely due to a disruption in the equilibrium of the subunit concentrations. Third, Rsp5 may only recognize a specific form of its substrate, e.g., a phosphorylated form. Therefore, when the amount of the substrate form is low compared to the total amount of that protein, the consequent stabilization of only the minor form in an *rsp5* mutant will not significantly affect the half-life measurement of that protein. Finally, Rsp5 may ubiquitinate a substrate at a specific point of the cell cycle. Thus, measurement of total protein turnover in an asynchronous population would obscure cell-cycle-specific changes for a particular protein. Similar observations have been made for some cyclin proteins (40). In fact, we observed that while the transcripts levels of *RFA1* increase during S phase, Rfa1 protein levels remain constant throughout the cell cycle (Fig. 6C and D). We also found that the degradation kinetics of Rfa1 protein does not differ significantly between S-phase-arrested and nonarrested cells (data not shown), suggesting that regulation is at the level of translation. However, we cannot exclude the possibility, as discussed above, that a critical subpopulation of the total Rfa1 protein is degraded in a cell-cycle-dependent manner, since we can only measure the total amount of the Rfa1 protein in our experiments.

Studies on the molecular genetics and biochemistry of both RP-A and Rad52 may help explain the ts phenotype associated with the two *rsp5* alleles in various genetic backgrounds. Recent work has shown that excess RP-A can actually inhibit DNA annealing in vitro (49, 52) and that Rad52 protein, which stimulates DNA annealing reactions (26, 52), can overcome this inhibition (49). Thus, *rad52 rsp5* strains may be ts due to an increased level of Rfa1 that dramatically inhibits spontaneous annealing reactions. In support of this, we also observed that *rad52 rfa1-D228Y* strains that overexpress *rfa1-D228Y* display a similar ts phenotype (unpublished data). For the *rsp5-26* allele, synthetic lethal interactions occur with any combination of *rad1*, *rad52*, *rfa1-D228Y*, or *rad51*. Although there are many possible explanations for this phenotype, perhaps an additional protein(s) is stabilized in the *rsp5-26* strain that inhibits recombination in the presence of these mutations. Alternatively, the stabilized protein(s) may lead to an increase in recombinogenic lesions, which would be detrimental in the absence of efficient DNA repair.

The spontaneous direct repeat recombination events assayed here in *rad1 rad52 rfa1-D228Y* strains likely proceed via single-strand annealing according to the following scenario (45). The first step is a double-strand break in the plasmid sequence, after which degradation of the 5′ ends leads to the formation of 3′ single-stranded DNA tails (46, 50, 60). These single-stranded tails participate in the homology search and subsequent annealing to promote the recombination event (10). In *rad52* strains, where annealing is greatly reduced, more-extensive degradation results in longer single-stranded 3′ DNA (60). It was demonstrated recently that the *rfa1-D228Y* mutation suppresses the formation of the long single-stranded 3′ DNA tracts normally found in *rad52* mutants (46). Therefore, we suggest that, in a *rad1 rad52 rfa1-D228Y rsp5* quadruple mutant, as a consequence of the prolonged half-life of Rfa1, the concentration of RP-A increases, allowing it to bind more extensively to single-stranded DNA. Consequently, this may reduce the ability of these DNA molecules to participate in the homology search and/or annealing reactions. This effectively reverses the recombination phenotype of the triple mutant (i.e., the increased recombination observed in *rad1 rad52 rfa1-D228Y* is reduced by the addition of an *rsp5* mutation).

At the onset of this study, we mutagenized a *rad1 rad52 rfa1-D228Y* strain to help define the "pathway" responsible for the observed increased recombination. For example, if the *rfa1-D228Y* mutation specifically activated an alternative recombination pathway, we expected to find mutations that subsequently reduced various steps. Surprisingly, none were found. Instead, our extensive screen to identify genes in this putative pathway resulted in the isolation of a single ubiquitination gene twice. Both *rsp5* alleles display synthetic lethality with *rad52* at a restrictive temperature and, furthermore, turnover of the Rfa1-D228Y protein is slowed in the *rsp5-25* background. These results argue that the *rsp5* mutations affect recombination by altering the cellular pathway involved in protein stability.

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